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EARLY DIAGNOSIS AND TREATMENT OF OPPORTUNISTIC
MYCOBACTERIAL INFECTIONS IN HIV-SEROPOSITIVE AIDS PATIENTS

MIDTERM REPORT

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<p>enzyme-linked immunosorbent assay)</p> <p>A highly specific (ELISA) system has been developed to detect circulating antibody in sera raised against the type-specific polar glycopeptidolipid antigens of <u>Mycobacterium avium</u>. These antigenic structures are unique to <u>M. avium</u>, and hence the purpose of the project was to exploit this knowledge to determine the prevalence of such antibodies in homosexual men at increased risk of HIV infection, and thus subsequent opportunistic infection with disseminated <u>M. avium</u> disease (human immunodeficiency virus).</p> <p>Progress within the project to date has consisted of extraction of a large number of serotype-specific GPL antigens, and conformation of their purity using thin-layer chromatography and GC/MS. The ELISA system was then initially tested for its specificity using a panel of monoclonal and polyclonal rabbit and mouse anti-GPL antibodies. Following completion of these evaluations, we then began to apply the diagnostic system to control sera</p>					
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(a large group of CSU graduate students) and to patients at Fitzsimons Army Hospital and at the Denver Public Health Center. Initial results from these screening procedures are presented in this report.



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Introduction

Members of the Mycobacterium avium complex (including M. intracellulare and M. scrofulaceum) are facultative intracellular bacterial parasites that until recently were more of interest to veterinarians than to physicians. That picture has rapidly changed in the last few years, however, in that M. avium infections have become the most prevalent cause of systemic bacteremia in patients with AIDS (1). It is now recognized that M. avium infections frequently occur in patients with AIDS, thus posing a significant clinical challenge (2).

M. avium infections are usually associated with constitutional disease such as fever, sweats, weight loss and wasting. The infection is systemic, with particular involvement in the bowel; in contrast, although pulmonary disease can also occur, its relative incidence is low. Drug therapy is difficult, in view of the resistance of most isolates to conventional anti-mycobacterial therapy.

The purpose of the current project was to apply serological methods to determine the incidence of antibodies to the type-specific glycopeptidolipid (GPL) antigens of M. avium in HIV-seropositive AIDS patients. We wish to determine if GPL-positive serology can be used as a diagnostic predictive marker for an increased risk of M. avium disease as the immunocompetence of the individual declines. As we will show here, a high incidence of GPL-positivity was detected in both HIV⁺ and HIV⁻ homosexual men, thus suggesting a high rate of colonization. This data may be of importance, in view of the rapidly improving control of other, more acute and life-threatening opportunistic infections.

Methods and Results

Mice. Specific-pathogen free C57BL/6 female mice were purchased from the Jackson Labs, Bar Harbor, Maine. They were immunized with type-specific GPL antigens

in Complete Freund's Adjuvant at the base of the tail. Following boosting, lymph node and spleen cells were harvested, treated with monoclonal anti-Thy 1.2 plus guinea pig complement, and then the remaining B cell-enriched cell suspension fused using polyethylene glycol to SP2/0 cells. Monoclonal antibody-secreting hybridomas were expanded following limited dilution and screened for reactivity for GPL antigens.

Bacteria. Members of the *M. avium* complex that were specific for GPL expression for each of the known serotypes were obtained from the authenticated collection held at the National Jewish Hospital, Denver. The identity of each isolate was further checked by means of seroagglutination and thin-layer chromatography. Mycobacteria were grown on 7H11 agar at 37°C. Rapidly growing strains were harvested at 7-11 days and slow growers after 21 days. Harvested cells were autoclaved, washed, and dried overnight in a P₂O₅-containing desiccator under vacuum.

Antigen extraction. Dried mycobacterial cells were lipid extracted by sonication in CHCl₃-CH₃OH (2:1, v/v) for 10 to 15 sec and heating in a 50°C water bath for 18h (3). These extracts were used directly or were treated with mild alkali (4).

As described previously (5), this procedure extracts cell wall-associated polar C-mycosidic glycopeptidolipids. Each set of GPL antigens obtained from the different serotypes carry individual characteristic short oligosaccharide chains linked to a relatively invariant fatty acyl-peptidyl-3,4-dimethylrhamnose core. Analysis of the oligosaccharide side chains has revealed that they consist of an invariant rhamnose/6-deoxytalose inner disaccharide, followed by a number of other sugars that comprise the antigenic variation of the molecule. Thus, as an example, the oligosaccharide of *M. avium* serotype 8 (one of the most prevalent serotype infections in AIDS patients) consists of 4,6-(1-carboxyethylidene)-3-methyl-D-glucosamine-1,3,-rhamnose-6-deoxytalose.

ELISA. Purified lipid extracts were dissolved in ethanol and then 50 ul samples applied to 96-well microtiter plates. Plates were dried overnight, and then blocked for 10 min with PBS containing 0.1% Tween 80. Patient sera diluted 1:100 were then added in a volume of 50 ul, and the plate incubated at room temperature for 30 min. The plate was then washed (x4) in PBS. Commercial peroxidase-linked anti-human IgG conjugate was then added (Cooper Biomedical; 1:1000 dilution) and the plate incubated for 45 min. After four additional washes, a chromogen substrate was added (0-phenylenediamine dihydrochloride in 30% H₂O₂ in citrate phosphate buffer, pH 5.0), and the plate incubated in the dark for 30 min. The reaction was stopped by the addition of 2.5N H₂SO₄ and read at 490 nm on an ELISA reader.

As a continuing quality control, we have periodically evaluated this ELISA system using our panel of monoclonal antibodies, and a panel of polyspecific rabbit antisera previously described (6). By these criteria, the assay system has worked well, with no cross-reactivity noted other than with those chemically similar GPL serotypes previously noted (6).

Results

1. Incidence of serum antibody to the type-specific GPL antigens in a pool of normal individuals. In an initial study we obtained 166 serum samples from graduate students attending the School of Veterinary Medicine at Colorado State University. The mean age of these students was 26; they were not questioned as to their sexual orientation.

Samples were run in triplicate against groups of GPL antigens (Table 1). Mean absorbance values were plotted as a scatter diagram, including upper 95% confidence limits for the gathered data (Fig. 1). Using this limit as a cut-off value, 2.4% of samples were high for antigen group A, 4.8% for group B, and 4.9% for group C (Table 2).

2. Incidence in HIV homosexual men. In a second experiment we selected at random 62 isolates from HIV gay men taking part in the Denver Public Health AIDS Management program. As shown in Table 3, 24% of samples were positive (that is, over the 95% confidence limit delineated by the control group values) for group A antigens, 40% were positive for group B, and 19% for group C.

3. Incidence in HIV⁺ patients. Using a serum bank at the Fitzsimons Army Medical Center, we have examined 90 isolates from patients who are known to be seropositive for HIV. To date we have only analysed reactivity against antigen group A (the "pathogenic" mycobacteria), but of these, 44% of patients showed heightened antibody reactivity to GPL antigens (Table 4). In further analysis, we broke down these values by age group (the thought being that older patients were on average likely to have harbored the virus longer) and compared each age group for helper T cell numbers, and GPL reactivity. As an additional control marker for humoral responsiveness, antibody to CMV was also included. As shown in Table 5, a general trend was observed between declining T cell numbers and increased GPL reactivity (from 34% to ~60%).

Conclusions

Heightened reactivity to the type-specific immunodominant GPL antigens of M. avium is not an indication of active disease; rather, it implies exposure to these environmental mycobacteria, and potential colonization. We show here that, in contrast to controls, patients attending the AIDS Management group in Denver, and the Fitzsimons Army Medical Center, have a much higher incidence of raised antibody levels to GPL antigens.

One of the curious aspects of M. avium infections in AIDS patients is that the disease is not usually primarily centered in the lungs, as might be expected. Instead, there is systemic involvement, particularly involving the gut. Thus, if the gut is the primary portal of both colonization and subsequent

dissemination of infection, then we are led to hypothesize that such colonization might come about by lifestyle practices not common to our control group. That GPL reactivity and active disease might come about as a result of increasing immunodeficiency is not supported by these findings. Instead, they suggest that lifestyle factors amongst the homosexual community appear to substantially increase the risk of exposure to colonization with M. avium.

We hope in the near future to have screened a fairly large serum pool, and so the implications of these results may continue to become clearer. However, as a preliminary midterm report, the diagnostic assay we have developed as the basis of this research program appears useful and should provide interesting, and perhaps provocative, information.

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Glycopeptidolipid serotype					
Mixed group A	1	2	4	6	8
	9	10	12	14	18
	19				
Mixed group B	3	5	7	11	13
	15	16	17	20	21
Mixed group C	22	23	24	25	26
	27	28	41	42	43

TABLE 1.
Allocation of mixed serotyping groups based
on natural distribution, chemical dissimilarity

Fig.1. Control data for GPL ELISA

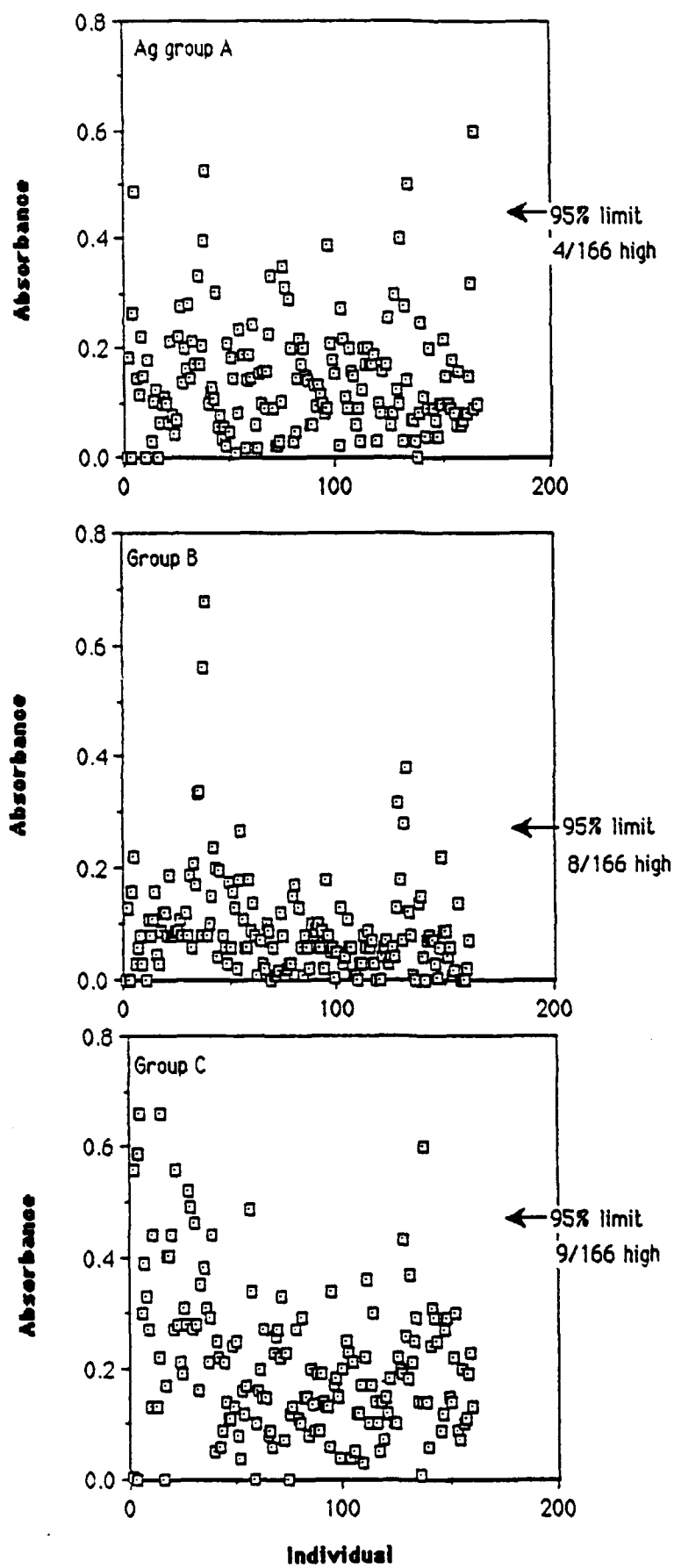


TABLE 2

Incidence of GPL+ sera in normal population

Ag group	n	high	%
A	166	4	2.4
B	166	8	4.8
C	166	9	4.9

TABLE 3

Incidence in Denver AIDS Management group

Ag group	n	high	%
A	62	15	24
B	62	25	40
C	62	12	19

TABLE 4

GPL reactivity in HIV+ Fitzsimmons group

mean T helper #	CMV Ab Titer	Ab to GPL group A
429+/- 227	85/95 (89%)	40/90 (44%)

TABLE 5

Breakdown of Fitzsimmons data by patient age

Age	T helper #	Ab to CMV	Ab to GPL (A)
25-28	486+/-206	29/31(94%)	11/32(34%)
29-32	484+/-218	33/38(87%)	12/30(40%)
33-36	295+/-129	8/10(80%)	7/11(64%)
37-40	453+/-356	15/16(94%)	10/17(59%)